UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, DC 20460

OFFICE OF
CHEMICAL SAFETY AND
POLLUTION PREVENTION

**MEMORANDUM**

Date: September 25, 2015

Subject: Protocol Review for Ottoman
EPA Reg. No. 777-113 (Protocol File Symbol 777-PA3 (DP #429159)

From: Alison Clune
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**Formulation from the Label: No formulation detailed on the proposed label. Protocol intended to be used for multiple products.**

## I BACKGROUND

The following submission is in response to the Efficacy Protocol Review dated April 7, 2014 for disinfection (bactericidal, fungicidal) claims on porous soft surfaces. As stated in the registrant's agent's letter dated May 27, 2015, the revised protocol addresses EPA's requests and also includes significant revisions to comply with revisions to the AOAC Use Dilution Method and/or Germicidal Spray Method (2013). The protocol was originally submitted under EPA Reg. No. 777-113 (Ottoman), however this has been changed to File Symbol 777-PA3 as the protocol is intended to support testing of several products with different formulations and forms. The data

package contained a letter from the registrant's agent dated May 27, 2015, the revised protocol (MRID 49646001), and a copy of the proposed label.

## II USE DIRECTIONS

The proposed product(s) is intended for use on soft surfaces as a bactericide and fungicide in residential environments. According to the proposed label the soft surfaces include household fabrics and soft furnishings. Directions on the label provided the following instructions for the preparation and use of the product on soft surfaces:

> **(Trigger Spray Application)**
> Pre-clean surfaces prior to use. Spray (the) (product) 2-3 (times) (pumps) (of the product) from a distance of 6 to 8 inches until fabric is wet. DO NOT SATURATE.
>
> **Fabric Disinfection:** For spot treatment only.
> **To Spot Disinfect Soft Surfaces (Fabrics):** Fabric must remain wet for [x] (minutes) (seconds). Let air dry. For difficult odors or heavy fabrics, repeat application.
>
> **(Aerosol Spray Application)**
> Pre-clean surfaces prior to use. Hold can (container) upright 6" to 8" from surface. Spray 2 to 3 seconds until covered with mist. DO NOT SATURATE.
>
> **Fabric Disinfectant:** For spot treatment only.
> **To Spot Disinfect Soft Surfaces (Fabrics):** Spray until fabric is wet. DO NOT SATURATE. Fabric must remain wet for [x] (minutes) (seconds). Let air dry. For difficult odors or heavy fabrics, repeat application.
> **To (kill) (control) (eliminate) (bacteria) (and) (fungi) on soft surfaces (fabrics):**
> Spray until fabric is wet. DO NOT SATURATE. Let air dry. For difficult odors or heavy fabrics, repeat application.

## III AGENCY STANDARDS FOR THE PROPOSED CLAIMS

No Agency standards apply.

## IV REGISTRANT'S RESPONSES TO EFFICACY DER CONCLUSIONS

1. Agency's Initial Response: Testing should be conducted with the independent lots of test substance all of which have a Certificate of Analysis verifying an active ingredient concentration at or below the Lower Certified Limit as accepted on the CSF. A lot that is ≥60 days old at the time of testing is not required.
   Registrant's Response: The protocol has been revised to indicate that the test substance formulation will meet these requirements and that a Certificate of Analysis for each batch will be included with the study report.
   Agency's Final Response: The issue has been adequately addressed. No additional information is required.

2. Agency's Initial Response: Application of the Test Substance: the specified volume of test substance to be applied by trigger/pump of 2-3 sprays/pumps is not an indication of volume to be applied to inoculated carriers. Indicate specifically the volume delivery

procedure including a range of potential variability based on multiple measurements of the chosen number of pumps per carrier as measured within a single bottle (e.g., what is the mean and standard deviation of 3 pumps volume measured 10-20 times from a single bottle as well as measurements determined across multiple different bottles.
Registrant's Response: The carrier treatment will follow the proposed Use Directions which is intended to simulate product use. The treatment will be precisely recorded in the raw data. This treatment method is identical to that used for all EPA registered aerosol or pump spray products. The variability present in a given delivery system will be demonstrated during the testing of replicate carriers and test lots.
Agency's Final Response: The protocol is based on the AOAC Germicidal Spray Test, which requires that carriers be sprayed for a specified time, distance from the carrier, and number of pumps, according to the label directions. A determination of the volume of the test substance applied is not necessary as the method corresponds to the label directions. No additional information is required.

3. Agency's Initial Response: The addition of organic soil to the test culture may not be specified as ≥5%. The target is 5% (v/v, within the tolerances of measuring instruments). An allowance for using serological pipettes has generally limit of what has been allowed for inaccuracy in this measurement.
Registrant's Response: The soil load has been changed to 5% (v/v) throughout the protocol.
Agency's Final Response: The issue has been adequately addressed. No additional information is required.

4. Agency's Initial Response: The inoculation of fabric carriers must be changed. The use of ≥0.01 mL is unacceptable. Five spots each 10 µL dispensed with a calibrated micropipettor (preferable positive displacement but minimally a calibrated micropipettor that has the volume range appropriate to dispensing 10 µL (i.e., 2-10 µL or 5-20 µL). One tip should be used to deliver all 5 spots and then changed for micropipettors and after every 10-20 or so for positive displacement pipettors.
Registrant's Response: The protocol has been revised to include the use of a calibrated positive displacement micropipette to inoculate 5 spots on each fabric carrier with 10 µL of the test culture. The pipette tip will be changed at least every 5 carriers.
Agency's Final Response: The issue has been adequately addressed. No additional information is required.

5. Agency's Initial Response: Carrier dry times, because a single test requires greater than 60 carriers and multiple tests are likely to be scheduled on a given test day (typically 1 organism, three lots, with carriers approaching 200), a window of time for a batch of dried carriers must be specified. See SOP for UDM testing at the OPP Microbiology Laboratory.
Registrant's Response: The protocol has been revised to indicate that all inoculated carriers should be used within 2 hours of drying.
Agency's Final Response: The issue has been adequately addressed. No additional information is required.

6. Agency's Initial Response: A description of the subjective metric(s) applied to the visual observation to determine that only dry carriers are used should be attempted at a minimum. This step cannot be standardized so a description if some form is essential. As currently presented in the protocol, the statement has almost no scientific value.
Registrant's Response: The protocol has been revised to describe the subjective observation of drying as follow: "If a visual observation is not sufficient in determining

whether a set of carriers is dry, an extra inoculated carrier can be used to blot the surface of a laboratory Kimwipe. A dried carrier will not transfer any culture to the Kimwipe (i.e. there will be no "wet spot" observed on the Kimwipe)."
Agency's Final Response: The issue has been adequately addressed. No additional information is required.

7. Agency's Initial Response: Because contaminated carriers are prepared in large batches, the number of carriers used to determine the carrier counts must be increased from 3 to 6 (but still in sets of 3). The first set of three carriers should be done when dried carriers are removed from the incubator and are first available. The second set of three are done at the end of tesing (but not to exceed the designate time window. All times should be recorded and tracked for compliance. The counts should be calculated independently for both sets of 3 carriers (beginning and end) and reported to the Agency both independently for initial set of 3 and the final set of 3 as well as combined weighted average for all 6 carriers which will be the single official control count reported for all tests conducted with that batch of carriers. It should be specified that an attempt to select a group of 3 carriers at random will be made.
Registrant's Response: The protocol has been revised to increase the number of carriers for dried recovery control counts to 6 randomly selected carriers per test organism per fabric type as recommended.
Agency's Final Response: The issue has been adequately addressed. No additional information is required.

8. Agency's Initial Response: The neutralizer volume change from 20 mL to 70 mL should be applied to any control experiments appropriate.
Registrant's Response: The protocol has been revised to specify a volume of 70 mL neutralizer to the appropriate controls.
Agency's Final Response: The issue has been adequately addressed. No additional information is required.

9. Agency's Initial Response: The physical transfer of exposed carriers and overspray to tubes of 70 mL of neutralizer (likely to be jars or similar container rather than a tube at the volumes specified and subsequent vortexing step) is undesirable for many reasons. The transfer fails to satisfy the requirements of a closed test system. Inefficient pipetting and volume loss of overspray significantly compound the high volume variability already introduced into the test system described in 2 above. These 2 significant sources of variability acting together must be assumed to have significant impact of test results (the transfer step of overspray is resource intensive and increases opportunities for the introduction of contaminants into the test system)
Registrant's Response: The method has been changed to treat, recover/neutralize, and incubate in a single vessel.
Agency's Final Response: The issue has been adequately addressed. No additional information is required.

10. Agency's Initial Response: With the more than tripled neutralizer volume proposed, is 5 g glass beads sufficient? The identity of the glass beads needs to be expanded to include size and tolerance if specified, material (i.e., soda lime, etc.), manufacturer, and approximate number of beads equivalent to 5 g for larger beads). The vortex speed should be estimated in generalized in ranges of high, medium, low, etc.
Registrant's Response: The use of glass beads has been removed in the modification of the method to satisfy Item 9.

Agency's Final Response: The issue has been adequately addressed. No additional information is required.

11. Agency's Initial Response: The tolerance for contamination in tubes, controls, plates, etc. must be addressed in the nonconformance section with specific tube numbers for test results and controls as well as on plates prior to testing.
    Registrant's Response: The protocol has been revised to indicate that contamination will be allowed in no more than 1 treatment vessel.
    Agency's Final Response: The issue has been adequately addressed. No additional information is required.

## V REGISTRANT'S RESPONSES TO EFFICACY DER RECOMMENDATIONS

1. Agency's Initial Response: The Agency review memorandum dated September 28, 2010 addresses the size of the carriers in the protocol (1'x1') compared with the label spot treatment area specified (2'x2'). On page 3 of the memo, the registrant indicated that to avoid confusion the 2'x2' phrase was removed from the label. The Agency concurred with that decision. The draft label submitted still states 2'x2' in the Fabric Sanitizer directions on page 7.
   Registrant's Response: The phrase has been removed from the proposed label.
   Agency's Final Response: The issue has been adequately addressed. No additional information is required.

## VI SYNOPSIS OF THE REVISED PROTOCOL

PURPOSE:
The purpose of this study is to demonstrate the disinfectant activity of the test substance against the specified test organisms as required for the desired claim on representative, soft, porous surfaces (i.e. cotton and polyester fabrics).

PROPOSED PROTOCOL OPTIONS:
- **Limited Efficacy Claims** either *Staphylococcus aureus* (ATCC 6538) or *Salmonella enterica* (ATCC 10708).
- **General (Broad Spectrum) efficacy claims** both *Staphylococcus aureus* (ATCC 6538) and *Salmonella enterica* (ATCC 10708).
- **Hospital or Medical Environment efficacy claims** *Staphylococcus aureus* (ATCC 6538) and *Pseudomonas aeruginosa* (ATCC 15442).
- **Pathogenic fungi** Trichophyton mentagrophytes (ATCC 9533). Additional strains optionally may be used including *Aspergillus niger* (ATCC 6275) *and Penicillium variable* (ATCC 32333)
- **Other microorganisms.** Effectiveness of disinfectants against specific microorganisms other than those named in the AOAC Use Dilution Method, AOAC Germicidal Spray Products Test or AOAC Fungicidal Test may be employed using this assay taking into account the alternate test requirements as stated in the guidelines.

The testing will be performed on two representative, soft, porous environmental surfaces representative of natural and synthetic fabric types (i.e. cotton and polyester fabrics). For the required strains above, 60 carriers/lot/test organism will be evaluated on 3 lots. For the additional microogranisms, 10 carriers/lot/test organism will be evaluated on 2 lots.

TEST SUBSTANCE IDENTIFICATION:

| **TEST SUBSTANCE FORMULA No.:** | | |
|---|---|---|
| Batch References (*) | Preparation Dates (**) | ExpirationDates (***) |
| Batch #1 | TBD | TBD |
| Batch #2 | TBD | TBD |
| Batch #3 | TBD | TBD |

TBD= To Be Determined

(*) Batch References:
The number of batches tested will be determined at the time of the test taking into consideration the claim desired. For example:

- Limited, Broad Spectrum and Hospital efficacy claims will require 3 batches of test product, whose active ingredient(s) comply with EPA Lower Certified Limit (LCL) Guidance (2013). Certificate of Analyses for each lot will be appended to the final report.
- Other microorganisms (e.g. fungi, bacteria) require 2 batches of test product.

(**) Preparation Dates:

- For Trigger / pump application products, this is the date in which the test sample is prepared for testing in the appropriate final packaging.
- For Aerosol application products, this is the date the bulk liquid is prepared. Additional information will be provided as to the date the test product was filled to the final packaging and the can pressurized.

(***) Expiration Dates:
This is the date applied to the finished product based on the chemistry data after which time the product cannot be used for additional registration claims testing.

DESCRIPTION/APPLICATION OF THE TEST SUBSTANCE:
The test substance is an liquid/aerosol spray/trigger/pump spray disinfectant. A description of the application of the test product and contact time will be described.
For example:

- For aerosol format products: A 2-3 second spray will be applied to the inoculated test surfaces from a distance of 6 to 8 inches for a ≤10-minute contact time.
- For trigger / pump format products: 2 – 3 sprays / pumps of the product will be applied to the inoculated test surfaces from a distance of 6 to 8 inches for a ≤10-minute contact time.

AOAC GERMICIDAL SPRAY TEST EXPERIMENTAL DESIGN / OPERATING TECHNIQUE

I. TEST SYSTEM - ORGANISMS:
The test organism(s) being used in the specific assay will be listed here including the appropriate identifier from the supplier (e.g. ATCC). For example:

1. *Staphylococcus aureus* (ATCC 6538)
2. *Pseudomonas aeruginosa* (ATCC 15442)
3. *Salmonella enterica* (ATCC 10708)

II. MEDIA, REAGENTS, AND EQUIPMENT
Including but not limited to the following:

1. Culture Broth – the appropriate culture/growth media for the organisms being tested will be listed here (e.g. nutrient broth, synthetic broth). For the required strains, this will comply with

the AOAC Use-dilution method (2013). For all other strains, this will be determined by the test organism being used and will be documented in the raw data. Refer to Table 1 for specific broth media requirements per organism.

2. Subculture / Neutralizing Broth – the subculture / neutralizing broth used in the assay will be listed here (e.g. letheen broth, DE broth, or any other broth known to neutralize the product). This will be determined by the test organism being used and the active ingredient type and it will be documented in the raw data.
3. Plating Media/Agar – the plating media / agar used in the assay will be listed here (e.g. tryptic soy agar, potato dextrose agar). This will be determined by the test organism being used and will be documented in the raw data. Refer to Table 1 for agar media requirements per organism.
4. Selective Media – the appropriate culture/growth media for the organisms being tested will be listed here (e.g. Mannitol salt agar, brilliant green agar, cetrimide agar). This will be determined by the test organism being used and will be documented in the raw data. Refer to Table 2 for the selective media requirements per organism.
5. Fabric Test Surfaces – Natural (i.e. 100 % Cotton). A description of the fabric, composition and source will be reported at the time of test.

1. Fabric Test Surfaces – Synthetic (i.e. 100% Polyester). A description of the fabric, composition and source will be reported at the time of test.
2. Horse Serum or other appropriate blood serum will be used as the bio-burden.
3. 70 to 99.9% Ethanol (EtOH) or equivalent
4. 0.05% isoocyylphenoxypolyethoxyethanol (TritonX-100)
5. $Na_2CO_3$
6. Forceps
7. Treatment vessel, sterile, 100 mL minimum volume capacity, (e.g. Corning 250 mL vessel with lid, or equivalent).
8. Water bath(s)
9. Bunsen Burner(s)
10. Incubator(s)
11. Refrigerator(s)
12. Freezer(s)
13. Hot plate or stove top burner
14. -70°C Ultra Low Freezer (for stock culture storage)
15. Fluke 52 K/J Digital Thermometer.
16. Rees Scientific Environmental Monitoring System.
17. Stopclock or other suitable timer that displays minutes and seconds.
18. Sterile, disposable pipettes
19. Sterile test tubes
20. Sterile disposable petri dishes
21. Sterile disposable needles/loops
22. Hemacytometer
23. Sterile Saline

## III. METHOD DESCRIPTION

### TEST FABRIC PREPARATION

In this assay, 1 inch by 1 inch fabric squares will be used in place of the hard, non-porous surface (i.e. glass carriers) to serve as the test surfaces to demonstrate disinfection on a soft inanimate porous surface.

If claims are desired for cotton fabrics, then the fabric squares used for testing will be made of 100% cotton. If claims are desired for polyester fabrics, then the fabric squares used for testing

will be made of 100% polyester. If general claims for fabric are desired, then both 100% cotton and 100% polyester fabric squares will be tested.

Two (2) examples of fabrics that may be tested are described below.

1. Natural fabric -plain weave 100% cotton fabric. The fabric will be completely desized, bleached and without bluing or optical brighteners. The source of the fabric will be identified at the time of testing. (i.e. Style 400M - obtained from Test Fabric Inc. Middlesex, New Jersey).
2. Synthetic fabric – 100% polyester fabric. The fabric will be completely de-sized, bleached and without bluing or optical brighteners. The source of the fabric will be identified at the time of testing. (i.e. Style XYZ – obtained from Test Fabric Inc. Middlesex, New Jersey).

Prior to testing, each fabric will be processed in the following manner. Each fabric type will be processed separately. The documentation of this processing will be recorded.

1. In a stainless steel pot, prepare a fabric scouring solution by adding 1.5 grams Sodium Carbonate ($Na_2CO_3$) and 1.5 grams Triton X-100 to 3 liters of deionized water.
2. Add approximately 100 grams of test fabric per liter of scouring solution.
3. Allow the solution to reach a rolling boil. Boil for ≥60 minutes but ≤70 minutes.
4. Using gloved hands and/or tongs, remove the fabric from the scouring solution.
5. Rinse thoroughly with deionized water until all traces (foaming) of the wetting agents are visibly noted to be gone. This can be achieved by running the deionized water to re-fill the pot. To aid in removing the scouring solution, the fabric can be rung out occasionally with gloved hands.

**Note: Only gloved hands should be used when handling the fabric from this point forward. This will avoid adding unwanted body oils to the fabric.**

6. Allow the fabric to air dry completely by hanging or draping, the time taken for drying will be recorded.
7. Using scissors and a ruler, cut 1 inch x 1inch test squares from the dried fabric.
8. Place each test square into separate glass petri dishes.
9. Steam sterilize
10. Cool and store the fabric test squares at ambient temperature for ≤30 days.

TEST SYSTEM PREPARATION (CULTURE TRANSFER)

The maintenance and storage of stock cultures is described in detail in Reckitt Benckiser SOPs. The procedure utilized for required bacterial strains follows the AOAC Use- Dilution Method (2013). The preparation of the Test System will only be described as per the below in this protocol.

BACTERIA:

1. For required strains, defrost a single cryovial at room temperature and briefly vortex to mix. Add 10 µL of the thawed frozen stock to a tube containing 10 mL synthetic or nutrient broth, vortex, and incubate at 36 ± 1°C for 24 ± 2 hour. Only one daily transfer is required prior to initiation of the final test culture. Additional bacterial strains may require alternate culturing techniques which will be detailed in the protocol.
2. For the final subculture step, inoculate a sufficient number of 20 x 150 mm tubes containing 10 mL synthetic or nutrient broth with 10 mL per tube of the 24 hour synthetic broth culture; incubate 48–54 h at 36 ± 1°C.

FUNGI:

3. For testing of pathogenic fungi, a spore suspension will be utilized as the inoculum for testing in place of the consecutive broth to broth transfers.
4. For fungi: Culture plates will be prepared in an appropriate vessel (e.g. either tissue culture flasks, Petri dishes, French square or Roux bottles which will be specified in the study protocol and raw data) by plating the inoculum from a stock culture onto the surface of Potato Dextrose Agar and incubating at 25 – 30°C for 10 – 15 days. The mycelial mats are removed from the surface of the agar by adding 5 g of sterile glass beads at 3 – 4 mm size and sterile buffered saline and shaking thoroughly. The spore suspension is then filtered through sterile glass wool to remove any hyphal elements. The density of the spore suspension will be determined through direct plate count and will be equivalent to ≥5 x 106 conidia / mL. The final method of fungal growth and harvesting will be detailed in the final report.
5. This suspension can be stored refrigerated for up to 1 month.

The target incubation temperature will be appropriate for the test organism and will be detailed in the protocol.

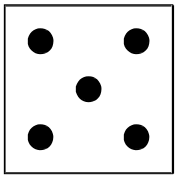
6. For the purpose of achieving the carrier count range, dilution of the final test culture may be performed using the sterile culture medium used to generate the final test culture (synthetic or nutrient broth).
    a. Dilution of the final test culture (e.g. one part culture plus one part sterile broth) should be made prior to the addition of the organic soil load (OSL) to the inoculum.
7. Concentration of the final test culture may be necessary in the event the bacterial titer in the final test cultures is too low.
    a. Concentration may be achieved using centrifugation (e.g. 5000 rpm for 20 minutes), and re-suspending the pellet in the appropriate volume of the sterile final test culture medium necessary to meet the carrier count range.
8. In addition, the use of a spectrophotometer to measure optical density (OD at 650 nm) is recommended to provide a tool (i.e. development of a standard curve) for assessing the need to concentrate or dilute the final test culture. Sterile broth medium should always be used to calibrate the spectrophotometer.

PREPARATION OF TEST CULTURE/ADDITION OF ORGANIC LOAD

1. Vortex the test culture for >3 seconds. For *P. aeruginosa,* prior to vortexing, the pellicle from 48 – 54 hour cultures must be removed from the broth either by decanting the liquid aseptically into a sterile tube, by gently aspirating the broth away from the pellicle using a pipet, or by vacuum removal.
2. Allow the culture to sit on the benchtop for 10 -15 minutes.
3. After this time, decant or pipet approximately the upper two thirds of each culture into a sterile vessel. Exercise care as to not transfer any clumps or debris from the bottle of the culture when decanting
4. Determine the amount of culture that will be necessary for testing purposes.
5. Pipette an appropriate amount of culture into a sterile vessel and add a volume of blood serum sufficient to achieve a final serum concentration range of 5% (v/v). Any combination of culture/serum volumes which results in a final concentration of 5% serum is acceptable (e.g. 19.0 mL culture + 1.0 mL serum for 5%. This will be referred to as the TEST CULTURE.
6. Any unusual observations or problems with the test culture will be noted on the raw data sheet (e.g. clumping, debris, graininess), and reported to the Study Director immediately after it is observed or discovered.
7. Repeat for each of the test systems.

FABRIC TEST SURFACE / CARRIER INOCULATION

1. Inoculate 0.05 mL (50-µL) of the test culture onto each sterile carrier using a calibrated positive displacement micropipette.
2. Ten (10) microliters (µL) of the Test Culture will be inoculated to each of five areas of the fabric carrier (the total inoculum of the five areas being 0.05 mL) using the same pipet tip. Tips should be changed at least between every 5 carriers. Each corner and the direct center are the target areas. See diagram below:



3. For **each fabric type**, inoculate the appropriate number of test squares for each organism and batch to be tested.
   For example:
   a. 60 fabric test squares per batch of the test substance for each test system to be assayed (3 test substance batches) (2 test systems) = 360 fabric test squares per fabric type.
   b. Two (2) test squares for (2) test systems to serve as Test Viability Control = Four (4) fabric test squares per fabric type.
   c. Six (6) test squares for each of (2) test systems to serve as Dried Recovery Count Controls = Six (6) fabric test squares per fabric type per organism.
   d. Additional carriers (e.g. 3 per organism per fabric type) should be inoculated to serve as extras or reserves in the event that a carrier is accidently dropped, or to assess if the inoculated carriers are sufficiently dry after the drying period. This is described in further detail in the following section (“Drying The Inoculated Test Surface”.

DRYING THE INOCULATED TEST SURFACE

1. Place the petri dishes containing the inoculated carriers into a relative humidity (RH) incubator that maintains 36 + 1°C and dry for 30 to 40 minutes. For *S. enterica*, dry the carriers for 10-12 minutes at 36+/-1°C.
2. The carriers will be visually observed at the end of the drying period to determine if the test culture is dried. If additional drying time is required, this will be documented in the raw data. If a visual observation is not sufficient in determining whether a set of carriers is dry, an extra inoculated carrier can be used to blot the surface of a laboratory Kimwipe. A dried carrier will not transfer any culture to the Kimwipe (i.e. there will be no “wet spot” observed on the Kimwipe).
3. At least 6 extra carriers per fabric type per organism will be dried to perform the Dried Recovery Control. Three of the carriers will be selected randomly and assayed before the treatment portion of the assay is performed, and (3) carriers will be selected randomly assayed after the treatment portion of the assay is performed. The purpose of this control is to quantify the number of bacteria surviving the drying process for each drying period.
4. Raw data entries at the time of testing will provide substantiation that the carriers were assayed before and after treatment as described above.
5. Use inoculated carriers within 2 hours of drying.

TREATMENT OF THE TEST SYSTEM (INOCULATED CARRIER) WITH THE TEST SUBSTANCE

1. The ambient temperature of the room will be constantly monitored and the maximum and minimum temperature recorded during the Treatment of the Test System and Subculture manipulations using an appropriate temperature monitoring device (e.g. Rees Environmental Monitoring System, Fluke Thermometer).
2. After drying, each inoculated and dried fabric swatch with be aseptically transferred from the glass petri dish to an individual sterile testing vessel. The inoculated side of the fabric swatch will be placed facing upward. The vessel will be re-capped until it is used in the efficacy test.
3. Prior to the treatment of an inoculated carrier, remove the lid or cap of the treatment vessel.
4. Tilt the treatment vessel slightly (equal to or less than a 45° angle).
5. Treat the inoculated and dried fabric carrier with a batch of the test substance as per the sponsors use instructions (e.g. 2-3 second spray from a distance of 6-8 inches from the surface, 2-3 pumps from a distance of 6-8 inches from the surface).
6. Replace the treatment vessel lid or cap to protect the test carrier from contamination.
7. At an appropriate time interval (±5 seconds), treat the next carrier as described in the previous steps.
8. Continue with Steps 3 to 6 until all of the inoculated carriers for that treatment set have been treated.
9. Allow the product to remain in contact with each inoculated fabric test square for the specified contact time (e.g. ≤10 minutes). The contact time starts at the time the product is first applied to the test square.

SUBCULTURE AND NEUTRALIZATION OF TREATED CARRIERS

1. Just prior to the contact time (e.g. ≤10 minutes) for the treated fabric test square, remove the lid or cap from the treatment vessel. At the contact time (± 5 seconds), add 70 mLs of neutralizer to the treatment vessel using a 100 mL sterile pipette. It is also acceptable to add a pre-measured 70 mL volume of neutralizer that was aliquoted prior to testing to the treatment vessel.
2. Replace the lid or cap of the treatment vessel.
3. Immediately vortex for 3-4 seconds each treatment vessel to ensure the treated test carrier is completely submerged in the neutralization media, and that the product overspray is thoroughly mixed with the neutralizer media.
4. Place all of the treatment vessels in the incubator at the temperature appropriate for growth of the test organism. The growth temperature will be detailed in the protocol, raw data and final report.
5. Incubate for the time specified for the test organism.

OBSERVATION OF SUBCULTURE TUBES AND RECORDING RESULTS

1. Remove the test materials from the incubator after the appropriate incubation period.
2. Observe each treatment vessel containing a carrier for the absence or presence of organism growth. Growth is indicated by turbidity. No growth is indicated by clear subculture media.
3. Record each subculture tube as “+” for growth, or “0” for no growth.

INVESTIGATION OF PRESUMPTIVE POSITIVES

6. In the event the evaluation of any batch for any of the organisms tested demonstrates a presumptive positive (turbid, cloudy tube), an investigation of the results will be conducted to verify the presumptive positive results. The investigation will identify the growth through

direct colony observation, gram stain and selective media as compared to a control. If necessary the growth will be identified to genus and species by an appropriate identification method (e.g. API, Vitek).

7. Results of the investigation will be reviewed by the Study Director and a conclusion will be drawn as to the identity of the presumptive positive, the acceptance or rejection of the results obtained, and any necessary next steps (e.g. re-test due to the confirmed presence of a contaminant).
8. Growth that is confirmed to be the test system is allowable in at least 1 of the 60 carriers treated (i.e. a result of 0/60 or 1/60 is a passing result). Therefore, if the growth in 1 treatment vessel is determined to be a contaminant (i.e. growth that was investigated, and concluded not to be the test system), and there is no growth in the other 59 treatment vessels, the assay will be accepted. This circumstance will be clearly identified in the final report.

**CONTROLS**

VERIFICATION OF THE IDENTITY OF THE TEST CULTURES

On the day of testing, the identity of each test system will be verified as per the instructions provided below. In summary, each test system will be streaked for isolation on an appropriate growth agar, streaked onto an appropriate selective media, and gram stained. Observations from these procedures will be compared to the known characteristics of each test system (e.g. for *S. aureus, S. enterica, and P. aeruginosa* 36 + 1°C for 48 + 2 hours. For fungi, *A. niger* incubate at 25 – 30°C for 5 - 7 days and *P. variabile* at 25 – 30°C for 10-15 days.

Any additional organisms and fungi will be detailed in the protocol and the results recorded in the raw data and reported in the final report.

For example (bacteria as detailed above):

1. After decanting, if needed, each test organism, aseptically perform an isolation streak onto TSA agar.
2. Aseptically streak each test culture onto the appropriate selective media as stated in the above table.
3. Incubate the growth and Selective Media Agar plates at the appropriate temperature for the same time as the test. The criteria stated in the incubation section of this protocol will apply to these test materials.
4. After incubation, examine the TSA plates for purity and colony morphology. Examine the selective media plates for type of growth (e.g. color of colonies) and any notable reaction of the agar media (e.g. color change, hemolysis). Record the observations.
5. From each TSA plate, select an individual isolated colony and gram stain. Record the observations.
6. Results of this verification will be reviewed by the Study Director. The identity of the test system will be accepted or rejected based upon the criteria provided in the table on the preceding page. This conclusion will be recorded in the raw data. If the test system verification is rejected, the assay will be considered invalid and the test will be terminated. All test materials will be discarded

SURVIVOR COUNTS / DRIED RECOVERY CONTROL CARRIERS

The purpose of this control is to determine the number of viable organisms that remain on the inoculated test carriers after the drying period. Six (6) carriers per test organism per fabric type are inoculated and dried as described previously in the test procedure. Dried recovery carriers are inoculated and dried along with efficacy carriers, and are selected randomly. Recovery counts

are performed on each dried recovery control carrier as described below. The survivor count procedure is described in detail in Reckitt Benckiser SOPs and will be detailed as per the below.

1. As stated previously, 3 of the inoculated Dried Recovery Control carriers will be dried and assayed before the treatment or efficacy portion of the assay is conducted, and 3 will be assayed after the treatment or efficacy portion of the assay is completed.
2. After drying, and at the appropriate subculture time, using an alcohol flamed and cooled forceps, subculture each inoculated and dried recovery control carrier into a treatment vessel that contains 70 mL of neutralizing media and 10 grams of glass beads. Vortex each treatment vessel containing a Dried Recovery Control carrier for 120 +/- 5 seconds.
3. Prepare tenfold or hundredfold dilutions from the treatment vessel containing each carrier in subculture media or an appropriate diluent. The treatment vessel containing the carrier is considered the $10^0$ dilution.
4. Using the pour plate technique, plate in duplicate 1mL and or 0.1 mL aliquots of the $10^{-1}$, $10^{-2}$, $10^{-3}$ and $10^{-4}$ dilution for each recovery control carrier with the appropriate media for the test organism.
5. Incubate the dried recovery control plates under the same conditions as previously described in the efficacy test. These conditions are specified in the incubation section of this protocol.
6. After the appropriate incubation duration, count the colonies on each plate and determine the number of organisms surviving on the carrier as per the instructions provided in the calculations section of this protocol.

TEST SYSTEM VIABILITY CONTROL CARRIERS

The purpose of this control is to confirm viable organisms that remain on the inoculated test carriers after the drying period. Two (2) carriers per test organism per fabric type are inoculated and dried as described previously in the test procedure.

1. After drying, individually subculture the two (2) inoculated test system viability control carriers into a treatment vessel that contains 70 mL of the subculture media that was used in the efficacy assay.
2. Incubate the treatment vessels under the same conditions as previously described in the efficacy test. These conditions are specified in the incubation section of this protocol.
3. After the appropriate incubation duration, observe each treatment vessel for the presence of growth. Growth or turbidity in each of the two (2) treatment vessels per test organism is considered an acceptable result for this control assay

NEUTRALIZATION OF THE TEST SUBSTANCE

1. The neutralization assay is performed using the same test substance parameters (dilution), test fabrics (100% cotton and/or 100% polyester), and treatment conditions (temperature, contact time).
2. For each organism dilution to be tested, treat two (2) sterile un-inoculated carriers with the test substance as described in the efficacy test. For example, 16 carriers would be treated when using each of the two test organisms, two fabric types, and two organism dilutions.
3. As in the efficacy assay, sterile fabric carriers are treated individually in treatment vessels.
4. At the contact time for each treated carrier, add 70 mL of the neutralizer media used in the efficacy test into the treatment vessel that contains the treated sterile carrier.
5. Serial dilute the test organisms (no organic soil) out to the 10-7 dilution.
6. For each organism and organism dilution tested, inoculate two (2) treatment vessels (containing the treated carrier and overspray) with 0.1 mL of the organism dilution that will deliver between 10 and 100 organisms into the subculture tube.

7. Confirm the number of organisms initially delivered into the subculture tubes by plating 0.1 mL for each of the test culture dilutions in duplicate.
8. Incubate the neutralization assay test materials under the same conditions as previously described for the efficacy test. These conditions are specified in the incubation section of this protocol.
9. After the appropriate incubation duration, observe each treatment vessel containing a carrier for the absence or presence of organism growth. Growth is indicated by turbidity. No growth is indicated by clear subculture media.
10. Record the result for each treatment vessel as "+" for growth, or "0" for no growth.
11. Count and record the number of colonies on each agar plate.
    Neutralization of the test substance by the neutralizer used in the efficacy study will be deemed acceptable if there is growth in both of the treatment vessels containing the treated carriers, and the number of organisms introduced into each vessel is determined to be between 10-100 colonies as demonstrated on the corresponding agar plates. The neutralization assay can be repeated with the same batch of subculture neutralizing media used in the efficacy test if the number of organisms is determined to be out of this acceptable range (i.e. <10 or >100 CFU).

MEDIA, TEST SURFACE AND ORGANIC SOIL STERILITY

The purpose of these controls is to verify that the media, test surface and organic soil used in the study on each assay date is sterile.

TEST SYSTEM INOCULUM COUNTS

1. Dilute each test system (without organic soil) to the $10^{-7}$ dilution.
2. Plate 1-mL aliquots of the $10^{-6}$ and $10^{-7}$ dilutions in duplicate with the appropriate growth agar. The plating agar will differ depending on organism type.
3. Incubate the agar plates under the same conditions as previously described for the efficacy test. These conditions are specified in the incubation section of this protocol.
4. After the appropriate incubation duration, count and record the number of colonies of each agar plate.

INCUBATION

The target temperature and duration for incubation of the test materials will differ depending on the test organism. The incubation temperature and time will be documented in the specific protocol, raw data and report.

For example:

- 36 + 1°C will be used for *Staphylococcus aureus* and *Pseudomonas aeruginosa* for 48 + 2 hours
- 25-30°C will be used for *A. niger* and *P. variabile*.

1. Incubation at a temperature outside of this range will be considered a protocol deviation, but will be deemed acceptable if the Dried Recovery Control values are within the expected range ($\geq 10^4$ organisms per carrier).
2. Additional instructions regarding incubation temperatures and duration will be followed and adhered to if necessary as documented in Reckitt Benckiser SOPs.

CALCULATIONS EXAMPLE:

1. Colony Forming Units (CFU) per ml (or organisms per ml)

   CFU/mL = A x B where

A= Dilution Factor. The dilution factor is the inverse of the serial dilution used.
B= Number of colonies per plate

2. MEAN LOG DENSITY (M) of DRIED RECOVERY COUNTS
    a. Count and record the number of colonies appearing on each plate. Record counts >300 as TNTC.
    b. For each replicate (duplicate plating), using values of 0 to 300, determine the 'Average Plate Count per Dilution' for each dilution plated. Round to the nearest whole number. If duplicate platings of a dilution yield results of 1 and 0, the average of these values (0.5) will be rounded to the nearest whole number (1).
    c. For each replicate, determine the 'Average CFU / mL' as per the following equation:

$$\text{Average CFU/mL} = \frac{(\text{avg. CFU for } 10^{-x}) + (\text{avg. CFU for } 10^{-y}) + (\text{avg. CFU for } 10^{-z})}{(10^{-x}) + (10^{-y}) + (10^{-z})}$$

    d. For each replicate, determine the 'Average CFU / carrier' by multiplying the 'average CFU/mL' by the volume of subculture medium in the test tubes containing the Dried Recovery Count slides.
    e. For each replicate, determine the Log Density by calculating the $Log_{10}$ value of the 'Average CFU / carrier'.
    f. Determine the Mean $Log_{10}$ Density (M) by averaging the individual Log Densities.
    g. Round results to provide 3 significant figures (e.g. $3.29 \times 10^4$ or 4.52 $Log_{10}$).
3. Rounding Instructions: Round off by dropping digits that are not significant. If the digit 6, 7, 8 or 9 is dropped, increase preceding digit by one unit; if the digit 0, 1, 2, 3 or 4 is dropped, do not alter preceding digit. If the digit 5 is dropped, round off preceding digit to the nearest even number: thus 2.25 becomes 2.2 and 2.35 becomes 2.4. These instructions are taken from the 19th Edition 1995 of Standard Methods for the Examination of Water and Waste Water, Part 1050 Expression of Results.

**PERFORMANCE CRITERIA**

ASSAY EVALUATION REQUIREMENTS:
The success criteria for soft surfaces will be identical to the EPA requirements for hard surfaces in the AOAC Germicidal Spray Test (2013). The success criteria is:

For bactericidal claims: The product must kill the required test microorganism on at least 59 of each set of 60 carriers. The requirement for soft surfaces includes both natural and synthetic fabric types. 60 carriers of each fabric type will be tested per test system per batch for required strains. Testing must be performed on each of 3 samples, representing 3 different batches.

For Fungicidal claims: Killing of the fungal spores on all of 10 carriers is required. The requirement for soft surfaces includes both natural and synthetic fabric types. 10 carriers of each fabric type will be tested per test system per batch. Testing must be performed on each of 2 samples representing 2 different batches per fabric type per test strain.

For Additional bactericidal/fungicidal claims: Killing of the test organism on all of 10 carriers tested is required. The requirement for soft surfaces includes both natural and synthetic fabric types. 10 carriers of each fabric type will be tested per test system per batch. Testing must be performed on 2 samples, representing 2 different batches per fabric type per test strain.

For Products requiring confirmatory data: In certain situations it is acceptable to rely on previously submitted efficacy data to support an application or amendment for a registration of a product and submit minimal confirmatory efficacy data to demonstrate the products ability to still be efficacious. Killing of the test organism on all of 10 carriers tested is required. The requirement for soft surfaces includes both natural and synthetic fabric types. 10 carriers of each fabric type will be tested per test system per batch. Testing must be performed on 2 samples, representing 2 different batches.

There is no statistical analysis of the test data.

ASSAY ACCEPTANCE CRITERIA:
The assay will be accepted for evaluating the test substance if the following criteria are satisfied:

1. The mean $Log_{10}$ density (LD) for each of the test systems (including fungi) must be at least 4.0 (corresponding to a geometric mean density of $1.0 \times 10^4$ CFU/carrier) and not above 5.5 (corresponding to a geometric mean density of $3.2 \times 10^5$ CFU/carrier).
2. The neutralizer must be shown to be effective, non-toxic, and support the growth of a low number of organisms (i.e. 10-100 CFU).
3. The identity of each test system must be verified on any day it is used for testing.
4. Growth must be present in the Test System Viability Controls.
5. The media, test surfaces and organic soil used in the study must be verified as sterile.

RETESTING GUIDANCE

1. For tests where the product passes, and the mean TestLD value is above $Log_{10}$ 5.5 log/carrier, no retesting is necessary.
2. For tests where the product fails, and the mean TestLD value is below $Log_{10}$ 4.0 log/carrier, no retesting is necessary.
3. For tests where the product fails and the mean TestLD value is above $Log_{10}$ 5.5 log/carrier, retesting may be conducted.

## VII AGENCY COMMENTS TO ADDITIONAL PROTOCOL REVISIONS

1. The Assay Acceptance Criteria and Retesting Guidance should follow the AOAC Germicidal Spray Test and/or OCSPP 810.2200 criteria for *Staphylococcus aureus, Pseudomonas aeruginosa, Trichophyton mentagrophytes,* and additional organisms. The stated acceptable mean $log_{10}$ density between 4.0 and 5.5 log/carrier should only apply to *Salmonella enterica*. For *Staphylococcus aureus* and *Pseudomonas aeruginosa*, the acceptable mean $log_{10}$ density should be between 5.0 and 6.5 log/carrier. For *Trichophyton mentagrophytes*, the acceptable mean $log_{10}$ density should be between 4.0 and 5.0 log/carrier. For additional organisms, the acceptable mean $log_{10}$ density should be at least 4.0 log/carrier.

2. In the section “Test System Preparation (Culture Transfer)”, step 1 should indicate that in the final subculture step for bacteria <u>10 µL</u> of the 24 hour culture should be transferred to the tubes containing 10 mL broth.